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**Occurrence of DNA methylation in *Daphnia magna* and
influence of multigeneration Cd exposure**

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Abstract

Most of the research on the epigenetic phenomenon of DNA methylation has been performed with vertebrates and plants. Knowledge on DNA methylation in *Daphnia magna*, a key test organism in aquatic toxicology, is completely lacking. Through epigenetic inheritance, effects of transient chemical exposure could be transferred to non-exposed generations, which could have a major impact on ecological risk assessment procedures. In this study, we determined if CpG methylation occurs in *D. magna* and if this can be influenced by exposure to toxic substances. Homologs of human DNA methyltransferases DNMT1, DNMT2 and DNMT3A were found in the partially available *D. magna* genome. Using an optimized “Amplification of Intermethylated Sites (AIMS)” technique, two methylated fragments were discovered in *D. magna* DNA. No homology was found for these sequences. The methylation and the *D. magna* origin of the fragments were confirmed with Southern analysis. This optimized AIMS technique was then applied to DNA of *D. magna* which were exposed to 180 µg/L Cd for two generations. Exposure resulted in a significant decrease in reproduction. The same methylated fragments with the same band intensity were observed in DNA of both non-exposed and exposed daphnids. As such, it could not be demonstrated that Cd exposure altered DNA methylation. However, the presence of DNA methylation in *D. magna* shows that potentially epigenetic effects may occur in this species.

50

51 ***Introduction***

52 Epigenetics is the study of changes in gene function that are mitotically and/or
53 meiotically heritable and that do not entail a change in DNA sequence (Wu and Morris
54 2001). One of the most studied epigenetic phenomena is DNA methylation, which is
55 the addition of a methyl group on the 5 position of cytosines in the DNA (Bird 2002;
56 Clark et al. 1994; Oakeley 1999; Watson and Goodman 2002). DNA methylation can
57 play an important role during development, cell differentiation, X-chromosome
58 inactivation and tumorigenesis (Santos et al. 2005). In mammals, methylation of DNA
59 in promoters is generally associated with silencing or reduced transcription of genes
60 (Bird 2002). It is hypothesized that CpG methylation represses transcriptional initiation,
61 not necessarily repressing transcription as such (Bird 1995). Recent research supports
62 this hypothesis for infrequently transcribed genes (Mandrioli 2007; Suzuki et al. 2007).
63 This explains the observation of methylation in transcribed genes. In *Arabidopsis*,
64 methylation has been found in a large number of moderately transcribed genes
65 (Zilberman et al. 2007). Methylated esterase genes in the aphid *Myzus persicae* were
66 shown to be actively transcribed and increasingly expressed (Field 2000), suggesting
67 that methylation plays a positive role in the expression of these genes. This could be
68 due to the above mentioned inhibition of spurious transcription.

69

70 Considerable research on DNA methylation and its function has been performed in
71 vertebrate organisms and plants (Chan et al. 2005; Reik and Dean 2001). Recently,
72 DNA methylation in invertebrates has received more attention. Some invertebrates
73 were long believed to have no DNA methylation at all, as suggested by (Urieli-Shoval

et al. 1982) for *Drosophila*. More recent research, however, has shown a low DNA methylation status in *Drosophila* (Lyko et al. 2000). The methylation was higher in early developmental stages compared to adults . It was not restricted to CpG dinucleotides, as is the case in most of the mammalian DNA methylation, but it was also observed in CpT and CpA dinucleotides. For other organisms, such as the nematode *Caenorhabditis elegans*, no methylation has been found up to now (Lyko 2001). Most invertebrates show a mosaic DNA methylation pattern, with roughly comparable amounts of methylated and unmethylated DNA (Simmen et al. 1999; Tweedie et al. 1997) as opposed to vertebrates in which a predominantly highly methylated genome is interrupted by unmethylated CpG islands.

Epigenetic changes can be triggered by environmental factors such as nutrition type or exposure to xenobiotics due to the dynamic state of the epigenome (Szyf 2007). A large proportion of offspring of mice that were fed with food high in folic acid, vitamin B12, choline, and betaine content during pregnancy displayed a high frequency of CpG methylation and heavily mottled and pseudo-agouti coat color. Conversely, a high number of offspring of mice fed with a control diet had a lower frequency of CpG methylation and a yellow or slightly mottled coat color (Waterland and Jirtle 2003). Similarly, exposure to toxic substances can also result in epigenetic effects. Exposing gestating female rats transiently to vinclozolin and methoxychlor caused reduced reproduction effects in the progeny which correlated well with altered DNA methylation patterns (Anway et al. 2005). This was observed up to four generations after exposure, indicating the possibility of inheritance of epigenetic changes, even

after only a short transient exposure to environmental stress. Epigenetic inheritance has also been demonstrated in *Drosophila* (Chong and Whitelaw 2004).

Through epigenetic inheritance, effects of transient chemical exposure could be transferred to non-exposed generations. Populations can thus experience effects of their ancestors' exposure. This could have a major impact on ecological risk assessment procedures.

For *Daphnia magna*, a key test organism in aquatic toxicology and an important species in many aquatic ecosystems, knowledge on DNA methylation is completely lacking. This research was aimed at determining whether CpG methylation is an epigenetic factor in *D. magna*, and if so, whether it can be influenced by exposure to toxic substances.

Materials and Methods

Sample collection and *Daphnia* cultures

D. magna Straus (clone K6) used in all our experiments was originally collected from a pond in Kiel (Antwerp, Belgium) and has been successfully cultured under controlled laboratory conditions for more than 10 years. One batch of 250 organisms was cultured as a control for four generations (F0 – F3) in M4 medium (Elendt and Bias 1990) slightly modified to reach optimal conditions for daphnids (Muyssen and Janssen 2004). A second batch of 250 organisms was cultured for two generations (F0 – F1) in the same medium with 180 µg/L of added Cd. Organisms were daily fed with an algae mix consisting of *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in a 3/1 cell number ratio.

Reproduction in *D. magna* in the control and Cd treatment was determined for two weeks following the procedures of OECD 211 guideline (OECD 1998), using ten replicates of individually held daphnids.

Algae-free juveniles of *D. magna* were obtained after 24 h from a batch of adults from the lab culture that was fed suspended kaolin clay particles (Imerys, UK) in an algae-free medium.

DNA extraction

DNA was extracted from 4 (juveniles), 21 or 27 (adults) day old daphnids with the MasterPure™ kit (Epicentre, Madison, WI, USA), following the protocol provided by the manufacturer. Fifty juveniles or 18-20 adult organisms were rinsed with deionized water, blotted dry and shock frozen in liquid nitrogen. The frozen organisms were subsequently transferred into a vial containing 300 µL Cell and Tissue Lysis buffer and 3 µL RNase A solution (5 mg/mL) and then manually homogenized with a microfuge tube pestle.

DNA was extracted from the algal mix in a similar manner. From the concentrated algal solution used as daphnid food, 300 µL was centrifuged for 10 min at 10000 g in a tabletop Hettich Mikro 200 R microcentrifuge (Andreas Hettich GmbH & Co KG, Tuttlingen, Germany). The supernatant was discarded, the algal pellet was shock frozen in liquid nitrogen and DNA was extracted using the MasterPure™ kit (Epicentre, Madison, WI, USA) as described for *D. magna*.

DNA integrity was checked on a 1.5% 0.5x TAE agarose gel and the DNA concentration was determined spectrophotometrically using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

AIMS

CpG methylation was investigated by a restriction-enzyme based method derived from the “Amplification of intermethylated sites (AIMS)” protocol described by (Frigola *et al.* 2002). However, we used tetracutters *HpaII* and *MspI* instead of hexacutters *SmaI* and *PspAI* (Wang *et al.* 2006). One microgram DNA was digested with 10 units *HpaII* (New England Biolabs, Ipswich, MA, USA) for 16h at 37 °C. This enzyme is sensitive to CpG methylation, hence it will not cut the restriction site if the inner C is methylated. The sticky ends are then filled in with 2 units Klenow enzyme (New England Biolabs, Ipswich, MA, USA) in the presence of a mix of 500 µM each dNTPs (Fermentas, St. Leon-Rot, Germany), by incubating for 15 min at 37 °C. This reaction was stopped by heating at 75 °C for 10 min. Subsequently, the DNA fragments were digested with 10 units *MspI* (New England Biolabs, Ipswich, MA, USA) by incubating for 6 h at 37 °C. This enzyme is insensitive to CpG methylation, hence it will cut regardless of the methylation on the restriction site. Adaptors were prepared by adding 50 nmol oligo “B” (5’-ATTCGCAAAGCTCTGA-3’) to 50 nmol oligo “B-GC” (5’-CGTCAGAGCTTTGCGAAT-3’) (Eurogentec, Seraing, Belgium) in a total volume of 1 mL, incubating the mixture at 65 °C for 2 min and then cooling it to room temperature for 30 to 60 min. Adaptors were ligated to the sticky ends created by the *MspI* digestion with T4 DNA ligase (New England Biolabs, Ipswich, MA, USA), by incubating with 800 cohesive end units T4 DNA ligase for 16 h at 16 °C. The DNA was then purified with the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Diegem, Belgium), following the manufacturer’s protocol. Adaptor ligated fragments were PCR-amplified using adaptor-specific primers, with the following PCR

conditions: 5 min at 95 °C, 35 cycles of (1 min at 95 °C, 45 s at 58 °C, 1 min 30 s at 72 °C), 6 min at 72 °C. The 15 µL reaction mixture contained 20 ng template (adaptor ligated fragments), 0.33 µM primer, 1.67 mM MgCl₂, 10 mM Tris-HCl (pH 8.8), 50 mM KCl and 1 unit of Taq DNA polymerase (Invitrogen, Merelbeke, Belgium). Selective nucleotides were added to the adaptor-specific primers (5'-ATTCGCAAAGCTCTGACGG-3'). Primers without extra selective nucleotides and primers with all possible sets of two extra nucleotides (i.e. 16 different primers) were tested.

In a modification of the method, two DNA subsamples of 1 µg were used. One subsample was digested with HpaII, while the other was digested with MspI. There was no fill in of sticky ends with Klenow enzyme. All other steps were performed as described above. A scheme of both the original and the modified AIMS methods is shown in Fig. 1.

Primers were labeled with $\gamma^{33}\text{P}$ -ATP (GE Healthcare, Diegem, Belgium) by polynucleotide kinase (Fermentas, St.Leon-Rot, Germany). Reaction products were analysed on large 5% denaturing polyacrylamide sequencing gels, cast and run in a Sequi-Gen GT Sequencing Cell (Bio-Rad, Nazareth, Belgium). Gels were exposed for two to four days to a sensitive film (Fujifilm, Düsseldorf, Germany) or for one or two days to an imaging plate, after which they were analysed by a FLA-5100 imaging system with MultiGauge Image Analysis software (Fujifilm, Düsseldorf, Germany).

Cloning and sequencing of AIMS fragments

Fragments of interest were excised from the polyacrylamidegel, reamplified by PCR, ligated into the pGEM-T vector (Promega, Leiden, The Netherlands) and transformed

into heat shock competent *Escherichia coli* DH5a cells. Transformed cells were selected on LB⁺ medium containing 100 µg/mL carbenicillin and checked for inserts by colony PCR using SP6 (5'-ATTTAGGTGACACTATAGAATACTCAAGC-3') and T7 (5'-TAATACGACTCACTATAGGGCGAATTGG-3') primers. Plasmids of positive colonies were isolated by miniprep (Birnboim and Doly 1979). Sequencing of the insert was performed at the VIB Genetic Service Facility, Antwerp, Belgium.

PCR on daphnid and algae DNA

The total 30 µl PCR volume consisted of 4 µl genomic DNA (2 ng/µl), 2 µl Forward primer (10µM), 2 µl Reverse primer (10 µM), 3 µl 10 x PCR buffer (Invitrogen, Merelbeke, Belgium), 2 µl dNTPs (10 mM), 0.2 µl *Taq* polymerase (5 units/µl, Invitrogen, Merelbeke, Belgium), 1.5 µl MgCl₂ (50 mM) and 15.3 µl water. Primers were based on the obtained fragments after the AIMS procedure (Fig. 3): 5'-GACTCGACCCATTCTGCAAT-3' and 5'-TTCGGAAGGGCTTTCCTTAT-3' as forward and reverse primer for the first sequence DMMF1; 5'-GGACGCGTGTACATTCACTG-3' and 5'-GGACCCATAAGACCCCTGTT-3' as forward and reverse primer for the second sequence DMMF2. The PCR program involved an initial denaturation for 4 min at 95°C, followed by 32 cycles of (1 min at 95 °C, 45 s at 54 °C and 1 min at 72 °C), and a terminal extension of 10 min at 72 °C. PCR products were separated on 1.5% agarose 0.5x TAE gels. The MassRulerTM DNA Ladder Mix (Fermentas, St. Leon-Rot, Germany) was loaded for reference. Ethidium bromide stained agarose gels were analysed with UV-transillumination using a GelDoc system (Bio-Rad, Nazareth, Belgium)

Southern analysis for confirmation of methylation

DNA fragments to be used as probe were amplified with PCR as described above and radioactively labelled with [α -³²P]-dCTP (Perkin-Elmer, Zaventem, Belgium) using Amersham Ready-To-Go DNA Labelling Beads (GE Healthcare, Diegem, Belgium). Non-incorporated nucleotides were removed with Micro Bio-Spin 30 columns (Bio-Rad, Nazareth, Belgium).

Digestion with MspI or HpaII was performed as described above, scaled up to 10 μ g of DNA. Digests were purified with the QIAquick Gel Extraction Kit (Qiagen, Venlo, The Netherlands) and run overnight at 25 V on a 15 x 15 cm 0.8% agarose gel in 0.5x TBE buffer. After electrophoresis, the gel was depurinated with 0.25 M HCl for 10 min, denatured (1.5 M NaCl and 0.5 M NaOH) for 30 min and neutralized (1.5 M NaCl and 0.5 M Tris-HCl, pH7.4) for 45 min. This gel was blotted on an Amersham HybondTM-N+ nylon membrane (GE Healthcare, Diegem, Belgium). After prehybridization in 5x SSC, 5x Denhardt's solution (1 g/L Ficoll, 1 g/L polyvinylpyrrolidone, 1g/L BSA), 0.5 % SDS (all chemicals from Sigma, Bornem, Belgium) and 100 μ g/mL denatured salmon sperm DNA (Invitrogen, Merelbeke, Belgium), the blot was hybridized overnight to 10 μ L of denatured radioactive probe (approximately 150 – 200 ng) in a solution with the same composition as the prehybridization buffer. The blot was rinsed cold in 2x SSC (0.3 M NaCl, 0.03 M sodium citrate) with 0.1% (w/v) sodium dodecyl sulphate (SDS), then consecutively washed at 60°C in 2x SSC, 1x SSC and 0.5x SSC, each time for 15 min. The membrane was exposed to an imaging plate for approximately 1 h after which this was analyzed by a FLA-5100 imaging system with MultiGauge Image Analysis software (Fujifilm, Düsseldorf, Germany).

Statistical and bioinformatic analysis

Significance of difference in reproduction between Cd exposed and non exposed organisms was tested using a t-test in Statistica (Tulsa, USA) with $\alpha = 0.05$. Basic Local Alignment Search Tool (BLAST, Altschul et al. 1997) analyses were performed at the National Center for Biotechnology Information (NCBI) server (<http://www.ncbi.nlm.nih.gov>) using BLASTn and both nucleotide collection and expressed sequence tag (EST) databases and at the *Daphnia* Water Flea Genome Database (<http://wfleabase.org>). For local BLAST searches ESTs of *D. magna* and *D. pulex* were downloaded from NCBI (May 2008). The sequences were cleaned using seqclean to remove contaminants, low quality and low-complexity sequences and subsequently clustered using TIGR Gene Indices Clustering Tool TGICL (Pertea et al. 2003). A set of unigenes was obtained by combining the contigs and singletons. The presence of *Daphnia* genes coding for DNA methyltransferase (DNMT) enzymes was investigated by searching the partly assembled *D. magna* genome for homologs of *Homo sapiens* DNMTs (DNMT1, A0AV63 / DNMT2, Q6ICS7 / DNMT3A, Q9Y6K1.4 / DNMT3B, Q2PJS8) using tBLASTn at the *Daphnia* Water Flea Genome Database. Sequences were analysed using the programs getorf and showorf embedded in the EMBOSS software package (Rice et al. 2000), <http://emboss.sourceforge.net/>).

Results

To detect whether *D. magna* is able to perform DNA methylation, we determined whether the genes essential for this process are present in this species. A search with tBLASTn in the partially assembled *D. magna* genome (version 2008_09) at the

Daphnia Water Flea Genome Database revealed unigenes with significant homology to DNMT 1, DNMT 2 and DNMT 3A (Table 1). These results indicate that *D. magna* possesses the molecular toolbox to perform DNA methylation.

After obtaining these indications for the occurrence of this type of modification, we aimed to unequivocally demonstrate the presence of DNA methylation in *D. magna*. To this end, *D. magna* DNA was analysed with the AIMS method. Amplification with non-extended primers resulted in a non-resolved smear both on agarose and polyacrylamide gels. Next, all 16 possible primers extended with two extra selective nucleotides were used. This resulted in a total of 899 clear and reproducible bands, with a nicely discernible pattern for most of the tested primers (Table 2). These fragments were considered as flanked by at least one methylation site. As a confirmation of the technique, the enzymes *MspI* and *HpaII* were used separately with two DNA subsamples and not sequentially with one sample (Fig. 1). Surprisingly, 897 out of 899 bands that were found following sequential restriction with *HpaII* and *MspI* were also detected, at a comparable intensity, in samples that were only restricted with the methylation sensitive *HpaII*. This probably indicates failed Klenow polymerization for those specific fragments (M. Jorda, personal communication). When Klenow polymerization fails filling up the sticky end of an unmethylated fragment after *HpaII* digestion, the adaptor is ligated to unmethylated fragments that will be amplified in the PCR together with the methylated fragments. All except two bands observed after restriction with *MspI* were also observed with comparable intensity after restriction with *HpaII*, indicating the absence of methylation at the restriction sites of these fragments.

Only samples amplified with a -GG extended primer resulted in two observed methylation specific bands on the polyacrylamidegel (Fig. 2). These bands were observed in samples that were treated with *MspI*, but not in samples that were restricted with *HpaII*. This observation was confirmed in three independently isolated DNA samples. The same procedure was executed with DNA taken from Cd-exposed juvenile organisms from the 2nd generation to investigate the extent of the effect of Cd on DNA methylation. Due to problems with the storage of the DNA samples, no analysis could be performed on the F0 Cd exposed generation. The significant difference between the total number of juveniles per surviving organism after two weeks of exposure of the Cd-exposed versus the reproduction of non-exposed organisms in the F1 generation (8 versus 27 juveniles per replicate, p-value 0.000001) confirmed the adverse effect of Cd at this concentration. Despite the clear effect at an organismal level, AIMS analyses resulted in the same band pattern (with the same methylated bands) in both Cd-exposed and non-exposed daphnids (Fig. 1).

The two “methylation specific” fragments DMMF1 and DMMF2 (*Daphnia magna* methylation specific fragment) were excised from the polyacrylamide gel, reamplified and sequenced (Fig. 3). The length of DMMF1 is 763 bp, whereas the length of DMMF2 is 620 bp. No significant similarities were found when both sequences were used in BLAST searches against the nucleotide collection database of NCBI (E-value cutoff = 0.10). Analysis of the sequences against the partial *D. magna* genome (version 2008_09) and the *D. pulex* genome (version 2006_09) using BLASTn on the *Daphnia* Water Flea Genome Database (<http://wfleabase.org>) also yielded no significant hits. Despite this inability to identify similar sequences in the partial genome or in a related genome, other elements indicate that the fragments might be coding for proteins. No

repeats were found in either sequence. DMMF1 exhibits a non-stop open reading frame. The longest open reading frame that can be detected in DMMF2 is 495 nucleotides. It was striking that no homology was found, despite the many *Daphnia* sequences available in the public databases, including the roughly annotated *D. pulex* genome . This observation might indicate that the detected specific fragments were amplified from a contaminating source rather than from *Daphnia*. The best candidate for this contaminant DNA is the algae mix used to feed *Daphnia* and which might still be present in the gut when the daphnids are lysed for DNA extraction. Specific primers were constructed on both fragments, and the primer pairs were used to amplify the sequences from DNA extracted from *Daphnia* and algae. Whereas a band was obtained with an expected size when DNA was used from *D. magna* or from juveniles that had not been fed, the fragment could not be amplified from algal DNA (Fig. 4). This finding thus demonstrated that the observed methylation specific fragments in the *Daphnia* DNA did not originate from the algae ingested by the daphnids. To confirm the methylation of the fragments, a Southern analysis was performed. DNA was extracted from a batch of juveniles that had not been fed and had not had contact with algae. One subsample was digested with HpaII, another subsample with MspI. Hybridization of the amplified parts of the methylated fragments resulted for each fragment in a band that was clearly visible in the lane with MspI digested DNA, but not in the lane with HpaII digested DNA (Fig. 5). This confirms the methylation of these specific fragments. The clear signal is a further confirmation that the fragments did originate from *D. magna* DNA.

Discussion

D. magna homologs of enzymes involved in DNA methylation were found in the partially sequenced *D. magna* genome. Three sequences showed a high degree of similarity to the sequences coding for human DNA methyltransferases (DNMTs). In a review of DNMTs in eukaryotes, DNMT2 is described as a strongly conserved and widely distributed protein, which was observed in 20 out of the 23 investigated species (Goll and Bestor 2005).

The AIMS approach confirmed the occurrence of methylation in *D. magna*. Two differential bands were detected. The methylation was confirmed by a Southern blot experiment in which the fragments were used as probe to hybridize against *D. magna* DNA digested with methylation sensitive or insensitive restriction enzymes. Two differential AIMS-bands correspond to a maximum of four methylated cytosines - one at each side of the fragments. However, the actual number of methylation sites will be higher since not all methylation sites can be detected with the applied AIMS approach. Small fragments will not be discernible in the lower part of the gel whereas long fragments will not be amplified under the PCR conditions used. Assuming that the fraction of methylated sites is the same for both the visible and “invisible” fragments

on the gel it is calculated that $\frac{2}{899} = 0.22\%$ of CpG sites in *Daphnia magna* are methylated, extrapolating methylation ratios for CCGG sites to methylation ratios for all CpG sites. However, the two fragments can be flanked by four different methylated restriction sites. Following the same reasoning, this thus results in 0.22% to 0.44% methylated CpG sites. The cytosine-methylation level in *Drosophila melanogaster* is

0.1% to 0.4% (Lyko et al. 2000), which is the same range as what is predicted for *D. magna* in this study.

Compared to cytosine methylation in other species this estimated degree of methylation is low. The percentage of methylated cytosines in the DNA varies considerably: 5% in mammals and birds, 10% in fish and amphibians, 0 to 10% in insects and more than 30% in some plants (Field et al. 2004). Little is known about DNA methylation in crustaceans. In *Artemia*, no detectable methylation was found using HPLC-analysis (Warner and Bagshaw 1984). In *Asellus aquaticus*, however, considerable DNA methylation was observed with an immunolabeling technique (Barzotti 2006).

In vertebrates, DNA methylation occurs almost exclusively in the context of CpG dinucleotides and also most invertebrate genomes exhibit methylation in a CpG context. However, it should be noted that also non-CpG methylation, e.g. CpA or CpT methylation has been described in plants, mammals and insects (Bernstein et al. 2007; Lyko et al. 2000). Since this type of methylation can not be detected with the AIMS method used in this study, it is possible that the total degree of methylation will be higher than predicted.

No relevant matches were found when the methylated sequences were used as query in BLAST searches against the partially sequenced *D. magna* genome, the fully sequenced *D. pulex* genome or the nucleotide collection database at NCBI. This suggests that these sequences are specific for *D. magna*. To obtain a general idea on the number of species-specific genes in *D. magna*, a BLAST search was performed of all unigenes from the *D. magna* EST dataset against the *D. pulex* EST dataset. With a cutoff E-value of 1×10^{-3} , 1567 out of 5003 unigenes resulted in no hit. This means that for approximately 31% of the currently known unigenes from the *D. magna* genome,

there is no similar gene in the *D. pulex* EST dataset. The latter dataset is considerably larger than the *D. magna* dataset and consists of 32030 unigenes. This 31% is an overestimation of the total fraction of *D. magna* specific genes. ESTs are always low quality sequences that are only fragments of genes. Moreover, ESTs are only available for genes that were expressed at the moment the EST database was created. It should also be noted that although the *D. pulex* EST dataset is rather extensive, it does not provide full coverage of all genes. This can be deduced from the relatively large number of 12413 singletons compared to 19617 contigs. Nevertheless, the fact that approximately 31% of the available *D. magna* unigenes had no similar counterpart in the *D. pulex* EST dataset indicates that there is a large amount of species-specific DNA in *D. magna*. This finding has also been reported for other congeneric species. For *Caenorhabditis briggsae* for example, more than 4% of the genes have no detectable matches in the related *Caenorhabditis elegans* (Stein et al. 2003).

Alternatively, the lack of sequence similarity could point to possible contamination by non-*Daphnia* DNA. However, the fact that fragments could be amplified from DNA obtained from juvenile *D. magna* that had not been exposed to algae, confirms that the fragments indeed belong to the *D. magna* genome and not to the algae DNA (food). This is further confirmed by the clear hybridization of the probes to the *D. magna* DNA on the Southern blot.

The effect of Cd on the daphnids' reproduction corroborates the results from previous studies with the same clone and same medium (Muyssen and Janssen 2004). For non-acclimated organisms and based on R_0 values (net reproduction), a 21-day EC_{10} and EC_{50} (95% confidence limits) of 167 (64-437) $\mu\text{g/L}$ Cd and 254 (191-336) $\mu\text{g/L}$ Cd, respectively, was calculated. For organisms acclimated to 150 $\mu\text{g/L}$ Cd for seven

generations, the 21-day EC₁₀ and EC₅₀ values (95% confidence limits) were 143 (66-311) and 230 (154-344) µg/L Cd. In the present study, a 70% reduction of reproduction was observed after 14 days at 180 µg/L Cd in organisms acclimated to 180 µg/L Cd for two generations. Exposure to chemicals has been shown to alter DNA methylation in several studies with mammalian cells. In vitro exposure of rat liver cells to 1.0 and 2.5 µM Cd in William's E medium caused hypomethylation after one week exposure and hypermethylation after 10 weeks of exposure (Takiguchi et al. 2003). Hypermethylation of DNA was also observed in human embryo lung fibroblast cells exposed to 1.2 and 1.5 µM Cd for 2 months (Jiang et al. 2008). Exposure of K562 cells to 2.0 µM of Cd resulted in hypomethylation of DNA after 24h and 48h (Huang et al. 2008). Similar results have been obtained with plants. In white clover and hemp, significant hypomethylation was observed after two weeks of growth in soils contaminated with Cd, Ni and Cr (Aina et al. 2004). In our study, no changes in DNA methylation pattern were observed in Cd exposed organisms. This suggests that Cd exposure does not affect the DNA methylation status of *D. magna*. However, it should be noted that not all CpG sites are visualized with the method used in this exploratory study. Consequently, a potential effect of Cd exposure on CpG methylation cannot be excluded. Other methods, in which a full genomic methylcytosine analysis is performed, could elucidate this further.

Apart from DNA methylation, there are other mechanisms of epigenetic regulation. Histone modifications such as acetylation, methylation and ubiquitination can regulate DNA transcription (Grant 2001; Zhang 2003). RNA interference (RNAi) comprises non-coding RNA sequences that can interfere with gene expression (Meister and Tuschl 2004). These mechanisms can possibly also be influenced by environmental

factors. Exposure of male rats to cyclophosphamide and mating them with non-exposed dams resulted in hyperacetylated histones in the pronuclei early after fertilization, which may lead to erroneous gene expression (Feil 2006). These kinds of effects are more difficult to investigate and literature concerning histone modification or RNAi after environmental stress is scarce.

In conclusion, this research has discovered DNA methylation in the genome of *D. magna* at CpG sites. The sequencing of the *D. magna* genome currently being performed at the Centre for Genomics and Bioinformatics (Indiana University, Bloomington, USA) will allow localization of the methylated sequences in the *D. magna* genome.

The methylated sites were detected in both Cd-exposed and non-exposed daphnids. No extra fragments were detected in DNA of Cd-exposed *D. magna*. As such, it could not be demonstrated that exposure to Cd altered DNA methylation. However, the fact that methylated DNA is observed, shows that potentially epigenetic effects may occur in *D. magna*. The role and consequences of epigenetics in environmental toxicity testing and ecological risk assessment certainly needs further investigation.

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565 Figure 1. Illustration of (A) the original AIMS procedure with one DNA sample and sequential digestions with both HpaII and MspI
566 restriction enzymes and (B) the modified procedure with two DNA subsamples and the single digestion with of either HpaII (B1) or MspI
567 (B2)

B2

Figure 2 – Detail of the fingerprint of two DNA-samples from *D. magna*: 4d old control juveniles (first two columns) and 4d old juveniles which have been exposed to 180 µg/L Cd for 2 generations (last two columns). HpaII = only HpaII digestion performed, MspI = only MspI digestion performed. Arrows indicate bands that appear in the samples on which MspI digestion is performed, but not in the samples on which HpaII digestion is performed. These indicate fragments which are methylated at the CCGG site.

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578 Figure 3 – Intermethylated sequences DMMF1 and DMMF2 in *D. magna*, reamplified
579 from the polyacrylamide gel. The sites of the internal primers for the PCR used to
580 confirm the origin of the sequences in the *D. magna* genome are underlined.
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584 Figure 4 – Results from the PCR performed to amplify the methylated sequences
585 (DMMF1 and DMMF2) from DNA of: (J) juvenile daphnids that have never been in
586 contact with algae, (Dm+A) daphnids that have been fed algae and (A) only the algae
587 that were used as food source during the culturing of the daphnids. M stands for
588 MassRuler™ DNA Ladder Mix (Fermentas, St. Leon-Rot, Germany).

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Figure 5 – Image of Southern blot, in which *D. magna* DNA that was digested with either the methylation insensitive restriction enzyme MspI or the methylation sensitive restriction enzyme HpaII was hybridized with ³²P-labeled DMMF1 (*Daphnia magna* methylated fragment 1) and DMMF2 (*Daphnia magna* methylated fragment 2) DNA probes. The scale denotes fragment length (number of nucleotides) as read from a Mass Ruler low range DNA ladder (Fermentas, St. Leon-Rot, Germany). Arrows indicate hybridized fragments in MspI lanes at the expected length of DMMF1 and DMMF2, absent in the HpaII lanes, confirming the methylation of the fragments.

599 Table 1 - *D. magna* DNA sequences showing homology with the sequences coding for
600 DNA methyltransferases in *Homo sapiens*

DNMT of <i>Homo sapiens</i> (accession number)	Homologous <i>D. magna</i> sequence	E value	Similarity at the protein level
DNMT 1 (A0AV63)	contig_885354	0.0	72%
DNMT 2 (Q6ICS7)	contig_1141364	3e-24	75%
DNMT 3A (Q9Y6K1.4)	contig_1436800	1e-13	52%

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606 Table 2 – Overview of the number of discernible fragments that could be detected on
607 the sequencing gel and the number of fragments that were detected as
608 methylated for each primer with specific extended nucleotides.

Selective nucleotides	Number of detected fragments	Number of methylated fragments
-AA	35	0
-AC	48	0
-AG	134	0
-AT	9	0
-CA	3	0
-CC	98	0
-CG	58	0
-CT	93	0
-GA	77	0
-GC	82	0
-GG	53	2
-GT	14	0
-TA	28	0
-TC	85	0
-TG	18	0
-TT	64	0

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